THE EFFECT OF RATE OF FREEZE ON THE STATE OF THE ACROSOME OF BULL SPERMATOZOA

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CHAPTER I

INTRODUCTION

The demand for greater utilization of superior sires has led artificial insemination organizations to look for the best and most efficient methods to handle and preserve semen. The increase in production by better techniques for storage, freezing and recovery of sperm cells would result in greater economic use of outstanding sires.

There has been much advancement in artificial insemination since Spallazani in 1780 reported the first successful artificial insemination of a dog. The organization of artificial insemination in the United States found its beginning in 1938. The semen from bulls in those earlier years was collected and stored at refrigerator temperature (5° C) but could be kept for only a few days. The greatest landmark in artificial insemination was the discovery of glycerol to aid long term preservation of semen by freezing at low temperatures of -79° C or -196° C.

The review of literature will show that much work has been done in the areas of storage, extenders, rates of freeze and other semen handling techniques. There has also been research to determine the best evaluator for use in predicting the fertility of spermatozoa. No single method has been found that can be used to reliably predict semen fertility.

The acrosome of the spermatozoa has in more recent years been studied for its role in fertilization. Most of the work in this area has been done concerning the morphological and functional importance of

the acrosome. In the boar, it is known that the integrity of the acrosome must be maintained in order for sperm cells to be fertile. Research with bull sperm cells has not clearly defined the relationship of acrosomal state to fertility.

It has been noted that the acrosome goes through an aging process and due to stress, the rate of aging seems to increase. Research has shown that freezing causes the loss of large numbers of sperm cells. Little information is known about the effect of freezing on the acrosome.

If the state of the acrosome is influenced by the rate of freeze, this could be of importance to the artificial insemination organizations in determining the most optimum rate of freeze. The research in this study was conducted to determine the effect rates of freeze may have on the acrosome condition of the bovine spermatozoa.

CHAPTER II

LITERATURE REVIEW

Evaluation of Semen Quality and Fertility

The use of artificial insemination and its rapid growth over the last few years has caused a continued search for reliable indicators of semen quality. Much work has been done to devise a method or a series of tests to be used to evaluate the semen and better predict its quality and fertilizing capacity.

Stone, et al. (1950) stated that a criterion for selecting semen samples to be used for artificial insemination which has a high correlation with the fertilizing capacity of the semen would be of great value. Initial motility of the spermatozoa, the whip like motion of the tail, has been the most widely and frequently used test for evaluating the semen quality. Reynolds (1916) was one of the first to describe the various types of motility and emphasized that vigor of motion was necessary for high fertility.

Swanson and Herman (1944) found a significant correlation ($r = 0.84$) between percent live cells and conception rate. Donham, et al. (1931) earlier reported a similar relationship between motility and conception rate. However, Ellenberge and Lohmann (1946) found little or no relationship between conception rate and motility where motility was rated on a scale of 0 to 100% in 10% increments.

Swanson and Herman (1941) determined that little improvement in

fertility occurred as motility increased beyond 50%. In artificial insemination organizations, semen with less than 50% motile sperm is not used. Initial motility alone has not been considered a satisfactory fertility index. Rate of movement is also another method to measure the viability of spermatozoa. Rate of movement is usually rated from 0-4 in 0.5 increments where 0 represents no movement and 4 the most rapid movement. Swanson and Herman (1944) and Margolin, et al. (1943) found a highly significant linear correlation ($r = 0.70$) between conception rate and the length of time a rate of movement of 2 or more was maintained in storage.

Volume of ejaculates has been evaluated as a predictor of fertility. It was found to be valuable in determining the total number of spermatozoa produced but no correlation with fertilizing capacity appeared to exist (Swanson and Herman, 1941; Ellenberger and Lohmann, 1946).

Concentration is widely used as an indication of fertility but has shown little predictive value in studies on semen of average or above average quality (Swanson and Herman, 1941).

The use of differential stains to determine semen quality has been used by different researchers. Shaffer and Almquist (1948) reported a highly significant curvilinear regression with fertility when using an eosin-analine blue stain to determine the proportion of living spermatozoa. However, the predictive value was not high with relatively good semen. Stone, et al. (1950) used the Fast Green FCF-eosine Y differential stain and found no within bull correlation between initial live spermatozoa and fertility. Lasley and Bogart (1943) used opal blue and employed the stain in their studies of artificial breeding in beef cattle. They reported a significant correlation of 0.41 between the

percentage of live spermatozoa and the motility rating of semen samples and a correlation of 0.83 between fertility and the percentage of live spermatozoa in diluted semen.

Other tests that have been investigated as indicators of semen quality are the methylene blue reduction test (Beck and Salisbury, 1943; Branton, et al., 1947), measures of metabolic activity of bull spermatozoa such as reduction of resazurin (Erb and Ehlers, 1950), cold shock test (Stone, et al., 1950; Kampschmidt, et al., 1953), and morphological abnormalities of spermatozoa (Saacke, 1970). These tests are variable in complexity and repeatability. To date, there is general agreement that no single test appears to be highly reliable, as a predictor of fertility. Various combinations of these tests are not reliable in revealing ejaculates or bulls of low fertility.

Since freezing and long term storage at -79° C or -196° C has become routine in artificial insemination, an even greater demand for measurements of the spermatozoa's freezing ability and its fertilizing capacity has arisen. Foote (1969) stated that motility is one of the most widely used test for semen quality, but motility should be noted as only one test for cell viability. Healey (1969) stated that most workers have used either a motility index or the ratio of live to dead sperm as a criterion of the success or failure of the freezing and thawing technique.

Motility evaluation of sperm cells in the bovine is one of the most widely used criterion for prediction of freezing and fertilizing potential. Pace and Graham (1970) stated that though this is generally true in bulls, motile sperm can be present in boar, turkey, and stallion semen after freezing and have little or no fertility. First (1961)

stated that motility of ram spermatozoa after freezing was approximately 45 to 50% but the fertilizing capability was low. Emmens (1962) obtained 50% post-freeze motility but very low fertilizing capability with frozen ram cells.

Niwa, Garrits, and Graham (1962) obtained 70% motile sperm after thawing frozen boar semen but no conceptions were achieved. Graham (1967) obtained a 40% post-freeze recovery but again no conceptions were achieved. Generally, boar spermatozoa frozen with glycerol has an acceptable percentage of motile cells (30 to 50%); however, fertilizing ability is extremely low. Hoffman (1959) reported only 1 litter from 11 inseminations using frozen semen of good motility. Baier (1962) obtained 3 conceptions from 43 inseminations with frozen semen. Graham, et al. (1971) felt that glycerol might be detrimental to the spermatozoa and reported 1 litter using an extender without glycerol. Pursel and Johnson (1971) reported 15 conceptions out of 23 gilts inseminated with a new extender when post-freeze motility was only 5 to 20%.

Extension of Semen

Basic Components of Extenders

Prior to 1939 diluents that were available were used only to extend the volume of semen for immediate breeding of a number of females. Phillips (1939) first reported that egg yolk had an advantageous influence upon bull sperm viability. He used an equal volume of egg yolk and phosphate buffer as a successful diluent for the preservation of bull semen.

The use of sodium citrate, to replace phosphate buffer, with egg yolk was later used (Salisbury and Knodt, 1947). They reported 3.6%

sodium citrate as the most satisfactory level and later Salisbury, et al. (1948) reported 2.9% sodium citrate dihydrate as equally desirable. The phosphate and sodium were compared to each other in regard to conception rates and proved to be very close. Swanson (1949) in determining the effect of varying proportions of egg yolk and sodium citrate buffer found that 3% citrate gave the highest motility. The 25% egg yolk gave the same motility as 50% egg yolk while 12.5% was inferior to either of the higher percent extenders.

The egg yolk in the diluent was early recognized as beneficial in protecting the sperm cells against cold shock (Easley, et al., 1942). However, Kampschmidt, et al. (1953) described the action to be due to the lipoprotein and lecithin found in egg yolk.

Sperm cells utilize glucose from the egg yolk. The additions of glucose to yolk diluents have increased the livability of the sperm cells (Salisbury and VanDemark, 1945). Martin (1965) stated that adding 0.1% fructose to the egg yolk dilution also increased longevity of the sperm cells. Bartlett and VanDemark (1962) found that sodium bicarbonate at 0.05 M supported better livability.

Whole milk is used as an extender and when properly boiled has given satisfactory results in sperm cell livability (Thacker and Almquist, 1951). Almquist and Thacker (1952) observed that the heated milk gave better fertility results with low fertility bulls than did the yolk citrate. Heated skim milk, another type of extender, when compared to yolk citrate had similar conception rates (Bolton and Durrel, 1954; Almquist, et al., 1954). When milk is used as an extender it must be heated because of the otherwise toxic effect to the sperm cells (Flipse, et al., 1954).

There are other extenders such as tris-buffered egg-yolk and Cornell University Extender (Foote and Dunn, 1962) that give acceptable conception results.

Role of Glycerol in Extenders

The extenders were used in early work to preserve semen at cool temperatures (5° C) for only a few days. Polge, Smith, and Parkes (1949) discovered that glycerol, when added to an extender, enabled sperm cells to be frozen and recovered without undue effect on the fertilizing capacity. In addition to altering ice crystal formation it appears to act as a salt buffer, minimizing electrolyte damage as water freezes out (Foote, 1969). Saacke and Almquist (1961) proposed that glycerol assists in maintaining the integrity of the membrane which is high in lipoprotein during cooling and freezing. Glycerol and the mechanism by which it exerts its beneficial effect on spermatozoa during freezing is not clear (Sherman, 1963). Lovelock (1953) indicated an intracellular action and stated that glycerol is a substance which will readily diffuse into the sperm cell. White (1957) also felt the protective action was intracellular. Sherman (1963) suggested that the protective action of glycerol may be extracellular.

The level of glycerol that gives the best protection to sperm cells has been well documented. Smith and Polge (1950) reported that glycerol levels of 10 to 15% in the extender gave excellent sperm survival rates whereas levels of 5% or lower gave lower survival rates. Graham and Marion (1953) found 10% glycerol in a 25% egg yolk extender to be superior to either 15 or 20%. Cragle and Myers (1954) concluded the optimum level of glycerol to be 7.6% when they studied extenders with glycerol

levels ranging from 2 to 14%. Saroff and Mixner (1955) when testing the relationship of egg yolk and glycerol content of extenders reported that an extender containing 20% egg yolk and 7% glycerol gave the best sperm survival after freezing. It is generally concluded by many researchers that the glycerol level for the optimum sperm survival is between 4-8% (Miller and VanDemark, 1954; Jones, et al., 1956).

The length of time (equilibration time) sperm cells are exposed to glycerol prior to freezing has been in question. It has been reported by several experimenters that holding of spermatozoa with glycerol at 5° C for a period of time improved the motility after freezing. Polge and Rowson (1952) recommended an equilibration time ranging between 15 to 20 hours when using an egg yolk extender. Cragle and Myers (1954) found 14.9 hours to be the most favorable equilibration time when using a time range from 4 to 28 hours. Since it is doubtful that they checked every 0.1 hour interval, the exactness of this number probably is erroneous. Miller and VanDemark (1954) comparing 2, 6, and 18 hour equilibration times, found the 6 hour period to be superior in terms of percentage of motile sperm cells surviving after freezing.

O'Dell and Almquist (1954) on the other hand using a heated skim milk extender did not observe any significant difference in sperm cell survival after freezing when using 0.5, 4, and 18 hour equilibration times. Later in 1956, O'Dell and Hurst compared 0.5 and 18 hour equilibration times and found the 0.5 time to be significantly better ($P < .05$). Berndtson and Foote (1969) added glycerol at 6 hours, 30 minutes, and 10 seconds prior to freezing and noted no significant difference in sperm survival rate among the three levels.

The length of time sperm cells must be exposed to glycerol before

freezing to have the best protection is still confusing. The protective action of glycerol before freezing seems to be achieved with only brief exposure.

Acrosome and Spermatozoa Function

Morphology of the Acrosome

The acrosome, also referred to as the head cap, is an anterior two layered membranous covering of the spermatozoa (Blom and Birch-Anderson, 1965). In the bull, the acrosome covers about 60% of the nucleus of the spermatozoa head. The remaining 40% is covered by the post-nuclear cap. Hancock (1953) described the acrosome as a compact refractile structure which in the living spermatozoa is closely attached to the surface of the sperm nucleus. The acrosome cap is labile to chemical or physical processes. The inner and outer acrosome layers, with the post-nuclear cap, form the equatorial segment.

The acrosome arises from the golgi apparatus during spermiogenesis (Clermont and Leblond, 1955). The acrosome is formed from the idiosome in the conversion of the spermatid to the mature spermatozoa which occurs in the seminiferous tubules of the testes. Austin and Bishop (1958) noted that the acrosome is a common structure in all the animal species. The size and shape of the acrosome, like the spermatozoa itself, differs among species. The acrosome is very large in the guinea pig, while it is very small in man and is intermediate in size in the domestic animal species (Bishop and Walton, 1960). Blom (1963) suggested that the acrosome played a double role; one as a carrier of necessary enzymes and as a protector of the sharp front edge of the nucleus of the sperm until the egg was reached.

Role of the Acrosome in Fertilization

Austin (1960) suggested that the acrosome was the source of the enzyme hyaluronidase. Hathaway and Hartree (1963) found a release of hyaluronidase to be associated with disruption of the acrosome. Hyaluronidase breaks down hyaluronic acid found in the intercellular matrix of the cumulus oophorus cells surrounding the egg. This concept was broadened to suggest that some lytic agent in the acrosome dissolved the zona pellucida, the outer covering of the egg (Srivastawa, Adams, and Hartree, 1965). The release of the lytic agent in the acrosome reaction would be instrumental in obtaining passage of the spermatozoon through this membrane.

Austin (1965) described the acrosome reaction as the normal alteration and eventual detachment of mammalian acrosome in the process of fertilization. Franklin, et al. (1970) suggested that an acrosome reaction is a prerequisite to sperm penetration of the zona pellucida. This is the reaction that is believed to make penetration possible after the sperm cell contacts the egg.

Bedford (1970) stated that purified hyaluronidase has no action on the zona substance, but since this coat is dissolved by adequate concentrations of trypsin (Chang, 1959) and by pronase, one might expect the hypothetical zona lysin to be a proteolytic enzyme. If a lytic enzyme is indeed responsible for passage of the sperm through the zona, then this enzyme must exist in close association with the inner acrosomal membrane. Franklin, et al. (1970) found in the Golden Hamster, that the function of the acrosome was to anchor the spermatozoa at the zona surface until the spermatozoa becomes capable of penetrating the zona. Hadek (1963) revealed that in rabbits, the acrosomal cap was intact at the onset of

zona pellucida penetration but became eroded away during passage of the spermatozoa through the layer. It should be pointed out that the morphological state of the acrosome and its relationship to fertility is still unclear. There is not total agreement that the state of the acrosome has an association with the fertility capability of the spermatozoa.

Abnormalities and Aging of the Acrosome

Blom (1963) suggested that in cases where the acrosome morphology is seriously distrubed i.e., the so-called knobbed sperm or knobbed acrosome, fertilization is impossible. Knobbed sperm have a thickened acrosomal projection which is folded back over the anterior end of the sperm head (Hancock and Trevan, 1957; Tiba, 1964; Bane and Nicander, 1966; and Saacke, et al., 1968). Bane and Nicander (1966) determined that the sperm that were knobbed were also sterile. The knobbed acrosome has been reported as a heritable trait (Donald and Hancock, 1953).

Saacke (1970) described the ruffled and incomplete acrosome abnormalities which in large quantities can cause subfertile bulls. Saacke, et al. (1968) reported a subfertile Holstein sire with high degree of incomplete knobbed and ruffled acrosomes. The sire had shown only a 40% 60 to 90 day non-return compared to the normal sires of 70% 60 to 90 day non-return. Three of four sons possessed the same types of abnormalities from the subfertile sire which were about 5% knobbed, 8% ruffled and 20% incomplete. The fertility of the sons was unknown. This does give some indication, however, that the acrosome abnormalities may be genetic and can be passed on to the next generation.

The change in the last few years has been that of separating genetic

abnormalities from normal aging processes. Saacke (1970) proposed that the aging sequence of the acrosome must be recognized as a normal process which all sperm undergo. The aging of the acrosome is the sequential alteration of the acrosome by various stages of deterioration resulting in its total detachment. Aging can also be stated as the degeneration of the acrosome.

The sequence of acrosomal alteration due to cell aging or injury appears constant, but the rate at which alteration occurs depends upon the ejaculate as well as semen handling techniques or sperm environment. Salisbury and Flerchinger (1967) stated that as normal spermatozoa age they also gradually lose the capacity to fertilize ova; but they lose at an even faster rate the functional integrity of the genetic information chain which is necessary to carry embryogenesis to normal partuation. Salisbury also observed that aging of frozen spermatozoa occurs more rapidly in summer collected semen than in semen collected from November to April. Salisbury and Flerchinger (1967) in an experiment where sperm were stored at temperatures of -79° C to -88° C, reported that while low temperatures depress the rate of movement of molecules and slow the appearance of the aging phenomena in the inseminated population, it does not prevent it.

Preservation of Spermatozoa by Freezing

The freezing of semen subjects the sperm cells to a high degree of stress. Even under the best freezing and handling methods it is common to have from 30 to 40% sperm death loss. Spermatozoa, like all tissues, contain water in various forms, some as free water and some as bound water intimately associated with the tissue colloids. When subjected

to cold temperatures crystal formation occurs in and around the spermatozoa. It is believed that the formation of large intracellular crystals of ice disrupt vital cellular associations and cause the death of cells upon freezing. The ideal method or rate of freeze would be to cause small crystals by freezing in one relatively homogeneous mass. A compromise rate of freeze to balance thermal and osmotic shock likely assists spermatozoa in surviving freezing (Bruemmer, et al., 1963; Yassen and Foote, 1967).

Polge (1953) stated that the temperature between -15° to -20° C was the critical range in which the most damage is done to the spermatozoa. The freezing of the extender from the liquid to the solid form takes place in this critical range. Polge felt if semen was cooled quickly enough over the space of about 2 minutes between -15° to -25° C, the spermatozoa are carried through the critical temperature range fast enough to avoid much damage. The semen at this time was stored in dry ice with a temperature of -79° C. Polge (1957) later suggested that slow cooling between -15° and -25° C increases the possibility of cell membrane damage through increasing the salt concentration as the water crystallizes. He found that -17° C is a critical temperature at which bull spermatozoa sustain great loss of motility when held for 10 minutes.

Methods to Evaluate Damage Due to Freezing

Nath and Patt (1970) when biochemically comparing unfrozen and frozen spermatozoa found that freezing causes physical disorganization, primarily by intracellular ice crystals. This could lead to enzyme leakage from the cell which can be determined by a number of processes.

Mann (1959) showed that cytochrome C is easily detached from the

sperm structure as a result of cellular damage or prolonged storage. Flipse (1960) reported that glutamic-oxaloacetic-tranaminase (GOT) and glutamic-pyruvic-tranaminase can be found in both seminal plasma and spermatozoa but in seminal plasma GOT was lower (Flipse, 1960; Roussel and Stallcup, 1965). Pace and Graham (1967) found that GOT and lactate dehydrogenase (LDH) increased in the extracellular media upon plunging spermatozoa into liquid nitrogen. The amount of GOT enzyme release is an indicator of cell stress or damage. Pace and Granham (1970) found a significantly higher release of GOT from frozen semen than fresh semen. They concluded that the amount of enzyme left in the cell after freezing is important to fertility.

The more enzymes remaining in the sperm following freezing indicates less sperm cell damage and a more viable sperm. The success of freezing and recovering sperm cells that have good (65%) fertility in some species and low or no fertility in others has led some researchers to look at the acrosome as a possible answer. Graham, et al. (1971) working with boar semen stated that while post-freeze motility of glycerolated spermatozoa was good (40%), most of the sperm cells had lost their acrosome. He further stated that his findings showed sperm cells without motility can still maintain a morphological integrity and may have the potential to fertilize eggs while the sperm cells with good motility may be damaged and be potentially ineffective in affecting fertility. Pursel and Johnson (1971) using a special extender for boar semen without glycerol only obtained post-thaw motility from 5 to 20%; however, they had successful (69%) conception compared to glycerol extenders where higher motility was achieved but with no conceptions. They indicated that acrosome morphology was a factor in obtaining fertility.

Rates of Freeze

O'Dell, et al. (1958) studied the effect of various freezing rates before and after -15° C upon the motility of spermatozoa during the freezing process and following storage for ten days at -79° C in heated skim milk diluent. His research indicated that the optimum freezing rate with fresh skim milk or homogenized milk diluents was 1° C per minute from +5° to -15° C, 5° C per minute from -15° to -50° C, and rapidly (approximately 20° C per minute) from -50° to -79° C. The optimum rate for semen diluted in 1:4 egg yolk-citrate-glycerol was 3° C per minute from +5° to -15° C and 5° or 10° C per minute from -15° to -50° C. He further showed that the greatest losses in motility occurred from -10° to -35° C. This was determined by removing the diluted semen at temperatures of -10°, -15°, -20°, -35°, -50° and -79° C. Samples thawed immediately after reaching -79° C showed the highest motility when a freezing rate of 10° C per minute from -15° to -50° C was used. However, following storage for ten days at -79° C there was a smaller loss of motile spermatozoa with a rate of 5° C per minute. Very rapid freezing (70° C per minute) was extremely detrimental; 75% of the total motile spermatozoa present before freeze were killed as compared to only 40% with a freezing rate of 10° C per minute from -15° C.

The rate of freeze can also be detrimental if it is too slow. VanDemark (1953) found that when using egg yolk-citrate-glycerol, .25° or 0.5° C per minute freezing rate to -79° C was inferior to 1° to 4° C per minute.

VanDemark and Kinney (1954) observed that cooling at a rate of 2° C per minute from +5° to -19° C and 4° C per minute from -19° to -79° C resulted in higher spermatozoa survival than cooling at a rate one

fourth as fast, or four times as fast.

Graham and Marion (1953) observed that a freezing rate of 2° to 3° C per minute seemed to be optimum when freezing bull spermatozoa in whole milk while 3° to 5° C per minute was optimum with the yolk-citrate-glycerol extender.

Erickson, et al. (1954) experimenting with antibiotics, levels of glycerol, extenders, and freezing rates stated that the best recovery with yolk-citrate was obtained by using both antibiotics and 7% glycerol and freezing over a critical period (-15° to -20° C) at 5° C per minute. With milk diluent the best recovery was obtained by using both antibiotics and 10% glycerol and freezing over the critical period at 2° C per minute.

Erickson and Graham (1959) using yolk-citrate and milk extenders used various rates of freezing to determine the optimum rate. They found yolk-citrate-glycerol gave the best spermatozoa survival at a rate of 3° C per minute from +5° to -15° C and 5° C per minute from -15° to -79° C. The semen extended in milk survived best when frozen at a rate of 2° C per minute from +5° to -30° C, and 5° C per minute from -30° to -79° C. The semen was stored at -79° C in dry ice and methanol. The 75 day non-return from 934 first services using yolk-citrate was 64.7%. The semen in milk extender from 945 first services had a non-return of 66.2%. This difference was not significant ($P < .05$).

The earlier methods of freezing, which have been previously mentioned, used dry ice to lower the temperature and adjust the freezing rate. The now simplier method for removing heat during freezing is to place the semen packages at 5° C into liquid nitrogen vapor (LNV). Forgason, et al. (1961) compared LNV freezing to dry ice alcohol (DIA)

bath freezing. The rate of freeze for DIA was 3° C per minute from +5° to -15° C and then was lowered 10° C per minute from -15° to -50° C. The rate of freeze for LNV was 2.5° C from +5° to -15° C and 6° C per minute from -15° C to -50° C as determined by thermocouple technique. The 68.3% spermatozoa survival rate with the DIA technique was not significant from 65.7% survival in LNV.

Roussel, et al. (1964) using a similar freezing rate for both LNV and DIA, found significantly greater loss of live spermatozoa ($P < .05$) when freezing in DIA (20%) than LNV (12.4%).

The present storage method of semen is in liquid nitrogen at a temperature of -196° C. Pickett, et al. (1961) and Bartlett and VanDemark (1962) stated the storage at -196° C in liquid nitrogen is preferable to the dry ice temperature of -79° C. Rapatz and Luyet (1966) stated that the colder temperature was preferred because extensive changes, including crystal growth, occurred at the warmer temperature.

Thawing of Semen

The two main parameters associated with the preservation of bovine spermatozoa are cooling rates and warming rates. Much experimentation has been done in the cooling rates as has previously been mentioned. The other factor associated with survival of frozen semen is the thawing process or warming rates. Rowson (1953) found no apparent difference when comparing survival rates of spermatozoa thawed in water at 38° to 40° C and those thawed at 5° C. O'Dell and Almquist (1954) and Brugman, et al. (1958) later concluded similar results. VanDemark and Sharma (1957) however observed significantly greater motility in samples thawed at 5° C as compared to 40° C. Hafs and Elliott (1954), Blackshaw (1955),

and Bruemmer, et al. (1963) reported that thawing bovine semen in water maintained at 38° to 40° C resulted in superior spermatozoa survival as compared to thawing at lower temperatures.

Pickett, et al. (1962) showed that in only 1 of 8 possible motility comparisons was semen thawed in ice water (2° C) significantly better than semen thawed at 40° C. Pickett, et al. (1965) later revealed from their studies using motility estimates, that thawing semen in ice water (1° C) was found to be superior to thawing in water maintained at 15° C. There was no significant difference between semen thawed in ice water (1° C) and 40° C, and both methods were better than any of the others used regardless of storage temperature.

Fertility is the final test of superiority of a thawing method. Dunn and Hafs (1953) observed no difference in 60 to 90 day percent non-return (% NR) between semen thawed at 40° C as compared to semen thawed at 5° C. Hafs and Elliott (1954) in a limited field of study noted thawing at 40° C was superior to 5° C and both were better than thawing at 20° C.

The reader will notice as in the case of freezing rates that there is a large amount of latitude as to the best thawing procedure to be used in handling semen. The reason for this the author believes may be in the variation among bulls as well as ejaculates.

There is general agreement that among bulls variation in ability of spermatozoa to freeze does exist. O'Dell and Hurst (1956) stated that there is considerable variation among bulls in the ability of their sperm cells to be frozen successfully. Buch, et al. (1956) also found a marked difference among bulls in the ability of their spermatozoa to withstand freezing although there were no significant differences

prior to freezing, as measured by motility. Steinback and Foote (1964) reported that semen from different bulls differed greatly in ability to withstand freezing.

It is evident that much work has been done to insure reasonable sperm cell survival post freeze. In view of the increasing concern over the role of the acrosome in reproduction, it will be necessary to re-examine several facets of current sperm cell preservation procedures to determine how the acrosome is affected.

This study was initiated to investigate the effect of various rates of freeze on:

1. morphological integrity of the acrosome, and
2. sperm cell livability.

CHAPTER III

MATERIALS AND METHODS

Semen Collection and Testing

Four dairy bulls from the Oklahoma State University herd were used in this study. The bulls were housed and managed similarly in the bull barn in individual pens. The bulls were ejaculated once a week on a routine basis prior to this study.

In semen collection a cow was restrained in the collection chute for use as a teaser animal. The bulls were led to an adjacent chute where the preputial area was cleaned with warm water and dried. The bulls were positioned behind the teaser animal in the collection stall for 5 to 8 minutes and then allowed to serve the artificial vagina containing 57° C water. The graduated collection tube of the artificial vagina was protected from cold shock by a plastic tube filled with 37° C water. An insulated jacket was placed around this plastic tube and collection funnel for insurance against cold shock. The semen tubes were kept in a water bath at 37° C throughout the time needed for initial evaluation and preparation of the stained slides.

The following data were obtained for each ejaculate:

(a) Semen Volume: Semen volume was obtained from the 15 ml. graduated tubes in which semen was collected to the nearest 0.1 ml. to determine if adequate amount of semen was available for freezing.

(b) Sperm Motility: This was estimated immediately after semen collection. A very small drop of fresh semen was mixed with a drop of 2.9% sodium citrate dihydrate solution on a prewarmed glass slide. The suspension was covered with a cover slip and examined with the light microscope (430X) with following determinations being made:

(1) the percentage of motile cells, expressed in units of 5 from 0 to 100,

(2) the rate of motility from 0 to 4 with the following rating:

0 No movement apparent (dead spermatozoa)

1 Sluggish or rocking motility typically showing no progressive movement

2 Progressive but slow and somewhat sluggish motility

3 Intermediate, fairly rapid motility

4 Maximum progressive motility, very rapid and vigorous.

The motility was used as the initial ejaculate percent live characteristic.

(c) Sperm Concentration: The density of spermatozoa per milliliter of semen was estimated according to the method described by Wells (1962). One-tenth ml. of the fresh semen was diluted in 4.0 ml. of 2.9% sodium citrate dihydrate solution at 37° C and mixed gently in the 17 mm. diameter tubular absorption cell. Percent light transmittance readings were obtained with a Cenco photelometer which had been standardized with

hemocytometer counts. These were converted to the concentration of spermatozoa per ml. of semen by reference to prepared tables. Appropriate dilution ratios were used to secure approximately 20 X 10^6 live spermatozoa per ml. after freezing with an estimated 35% death loss occurring during freezing. Ejaculates that were exceptionally dilute or that had either sluggish motility or fewer than 50% live spermatozoa were not used in this study.

Processing and Handling of Frozen Semen

The extender used in this study was prepared fresh for each trial and consisted of 20% egg yolk, 80% sodium citrate (2.9%) solution, 500 mcg of streptomycin, and 500 I.U. of penicillin.

The fresh semen was diluted in the egg yolk-citrate extender to obtain an initial volume of 40 ml. The 40 ml. of the extender with semen was placed in an Erlenmeyer Flask that was in a beaker containing 36° C water. The beaker was then placed in the refrigerator for gradual cooling to 5° C.

The extended semen was allowed to cool for 2 hours. At this time glycerol, a cryoprotective agent, was mixed with the extended semen to give a final level of 6%. The glycerinated extended semen was placed in 1 ml. ampules and heat sealed with a hand sealer. The ampules were placed on canes, 5 canes for each bull, after the extended semen had been in the cooler for 4 to 5 hours. The canes of ampules were then frozen in liquid nitrogen vapor.

The method of freezing was to place 3 inches of liquid nitrogen into a 20 X 15 inch styrofoam basket. A wire rack was made to hold the

canes of ampules and devised to control the level the ampules could be kept above the liquid nitrogen. Two low scale centrigrade thermometers were placed in the wire rack with the ampules to record change in temperature. The temperature was recorded in 1 minute intervals.

In this split ejaculate study, 1 of the 5 canes of ampules from each bull was subjected to one of the following 5 different rates of freeze. The rates of freeze used in this study ranged from slow to accelerated rate with the most optimum rates included for comparison. The rates of freeze were:

(1) Slow — .5° per minute from +5° to -30° C then 3° per minute from -30° to -50°C

(2) Moderate — 3-5° per minute from +5° to -50° C

(3) Intermediate — 5° per minute from +5° to -30° C then 20° per minute from -30° to -50° C

(4) Rapid — 10° per minute from +5° to -50° C

(5) Accelerated — 20° per minute from +5° to -50° C

All were lowered from -50° to -80° C at 20° per minute and then transferred to a liquid nitrogen tank.

Twelve to 15 hours later the ampules were thawed, 4 to 6 at a time, in ice water (1° C). The ampules were left in the ice water until they became liquid (8 to 10 minutes). The ampules were then unsealed and placed in plastic centrifuge tubes and centrifuged for 5 minutes at 500 RPM. The supernatant was poured off and 0.5 ml. of warmed (37° C) sodium citrate was added. The pellet of sperm cells was mixed thoroughly and recentrifuged for 3 minutes. The supernatant was again poured off and .25 ml. sodium citrate was added. This was mixed and then centrifuged for 2 minutes. After the 2 minutes, the supernatant was again poured off and .2 ml. of sodium citrate was added and mixed. The sus-

pended spermatozoa had been properly washed and ready to be stained with the acrosome stain and the nigrosin-eosin live-dead stain. Previous experience had shown that this procedure was necessary because spermatozoa suspended in the egg yolk-citrate-glycerol extender did not stain well.

Staining Procedure

Acrosome Staining Technique

Duplicate slides from all initial ejaculates and all post-thawed semen were prepared with the acrosome stain (Wells and Awa, 1970) for studying acrosome characteristics. The fresh semen was stained immediately after collection and the frozen semen was stained following the centrifugation procedure described earlier in this chapter. The stain was prepared as follows:

Solution "A": Consisting of a 1% solution of water-soluble eosin B (total dye content 88%) in glass-distilled water.

Solution "B": Consisting of 1% solution of water-soluble Fast Green FCF (total dye content 90%) in glass-distilled water.

On the day of semen collection one volume of solution "A" was mixed with two volumes of solution "B" and 1.7 volumes of ethyl alcohol. Stock solutions "A" and "B" were prepared monthly and stored at 5° C. Sperm cell smears from the initial ejaculates were made, stained and examined as follows:

1. One-tenth milliliter of fresh semen was diluted in 0.9 milliliter of 2.9% solution of sodium citrate in a prewarmed 17 mm. diameter tubular absorption cell. The dilution rate was

narrowed in certain cases when the concentration of spermatozoa in the ejaculate was low.

2. Two drops of this suspension were withdrawn with a prewarmed dropper and added to four drops of the acrosome stain in another similar prewarmed tubular absorption cell.
3. One to two minutes later on the fresh ejaculate and four to seven minutes on the post-freeze sample, one small drop of the sperm-stain suspension was withdrawn and placed on a prewarmed clean microscope slide, and then smeared in a thin layer.
4. The smears were air-dried at 37^{o} C and a glass cover slip was mounted with diaphane.
5. The semen supply, diluting and staining solutions and slides were maintained at 37^{o} C.
6. Two hundred sperm cells were examined on each slide to determine the acrosome condition. This was accomplished with the light microscope under oil, at a magnification of 970X, and using a blue filter.
7. The acrosome condition was recorded as nonaged or aged. The nonaged sperm cells were those that displayed a uniformly smooth acrosome that was closely adherent to the nucleus. Aged acrosomes were those that exhibited any of the following morphological changes:
   (a) Elevated or Thickened Acrosome: This is characterized by a partial or complete swelling of the outer membrane of the acrosome cap and thus an enlargement in acrosomal size.
   (b) Rough Acrosome Cap: This is characterized by an irregular

surface either over the anterior portion of the cap or spreading over the entire surface of the acrosome.

(c) Enlarged Equatorial Segment: This is characterized by the typical half moon shape of the equatorial segment becoming exaggerated as the acrosome becomes loosened from the sperm cell head.

(d) Detaching Acrosome Cap: The acrosome is in the process of loosening from the sperm head, eventually leads to the capless sperm state.

(e) Disintegrating Acrosome Cap: This indicates the latter stage of aging.

(f) Capless Sperm: This is characterized by the lack of the acrosome and is considered the terminal stage of the aging and/or deterioration sequence. The upper portion of the nucleus stains a light pink instead of the green which is typical when the acrosome is present.

Live-Dead Stain

The live-dead stain is a differential stain which stains the dead sperm cells while the live sperm cells do not take the stain. The stain used in this study was the nigrosin-eosin live-dead stain as described by Hancock (1952). The live-dead differential stain was prepared by dissolving 30 grams of water soluble nigrosin and 5 grams of eosin Y (total dye content 92%) in 300 milliliters of double distilled water. The nigrosin is used to provide a suitable background to facilitate recognition of live from dead sperm cells while the eosin Y stains the dead sperm cells red.

Spermatozoal smears were prepared by placing 1 drop of semen into 5 to 8 drops of nigrosin-eosin stain and mixing gently. Duplicate slides were made by smearing one drop of the suspension on prewarmed glass slides.

The live-dead stain was used only on the post-freeze samples. Two-hundred sperm cells were examined on each slide to determine the percent live. This was accomplished with the light microscope at a magnification of 430X. The slides in this study were number coded using a random digit table. This was used to prevent the person doing the slide evaluation from knowing the rate of freeze of which he was evaluating.

Semen was collected from 4 bulls on 5 different collection days. The ejaculates were split into 5 parts and each was subjected to 1 of 5 different rates of freeze. Differences among bulls, dates and rates of freeze on percent aged acrosomes and percent live sperm cells were examined for significance with an analysis of variance procedure using a modified factorial design. An ejaculate from a given bull on a given date was considered the main plot and the rates of freeze the treatments. Orthogonal comparisons were used to test the means in this study. Bull X rate, bull X date, and rate X date interactions were an integral part of the analysis. If significant, these interactions will not allow unqualified statements about the bull, rate, or date differences. If there are no significant interactions, then the three way interaction of bull X rate X date can be used as the error term to test significance of bull, rate, and date effects. However, significant interactions will only allow the use of the two way interactions as error terms to use in testing significance of bull, rate, and date. The conclusions concerning the effects of bull, rate, and date will of necessity be modified by the existence of interactions among these factors.

CHAPTER IV

RESULTS AND DISCUSSION

The four bulls in this study were collected with an artificial vagina and each ejaculate was treated as a population of sperm cells. The five rates of freeze were imposed on each ejaculate. The particular rates of freeze were chosen for the study in order to obtain different degrees of stress along with the optimum rate. The slow rate (.5° C per minute) and accelerated rate (20° C per minute) served as the extremes. The moderate rate (3° to 5° C per minute) was the recommended rate now most in use (Erickson and Graham, 1959). The rapid rate (10° C per minute) was a medium rate devised between the slow and fast rates.

The intermediate rate (5° C per minute to -30° C; 20° C per minute to -80° C) was used to observe the effect of freezing past the critical range and then increasing the rate of freeze. Polge (1957) stated that the critical range was -15° to -25° C when the phase change from liquid to solid takes place in the extended semen. The rates of freeze are seen graphically in Figure 1.

The laboratory procedures and semen handling were considered uniform and all samples were treated as nearly alike as possible with the only exception being the different rates of freeze.

Percent Live Evaluation

As can be seen in Figure 2, all rates of freeze drastically reduced

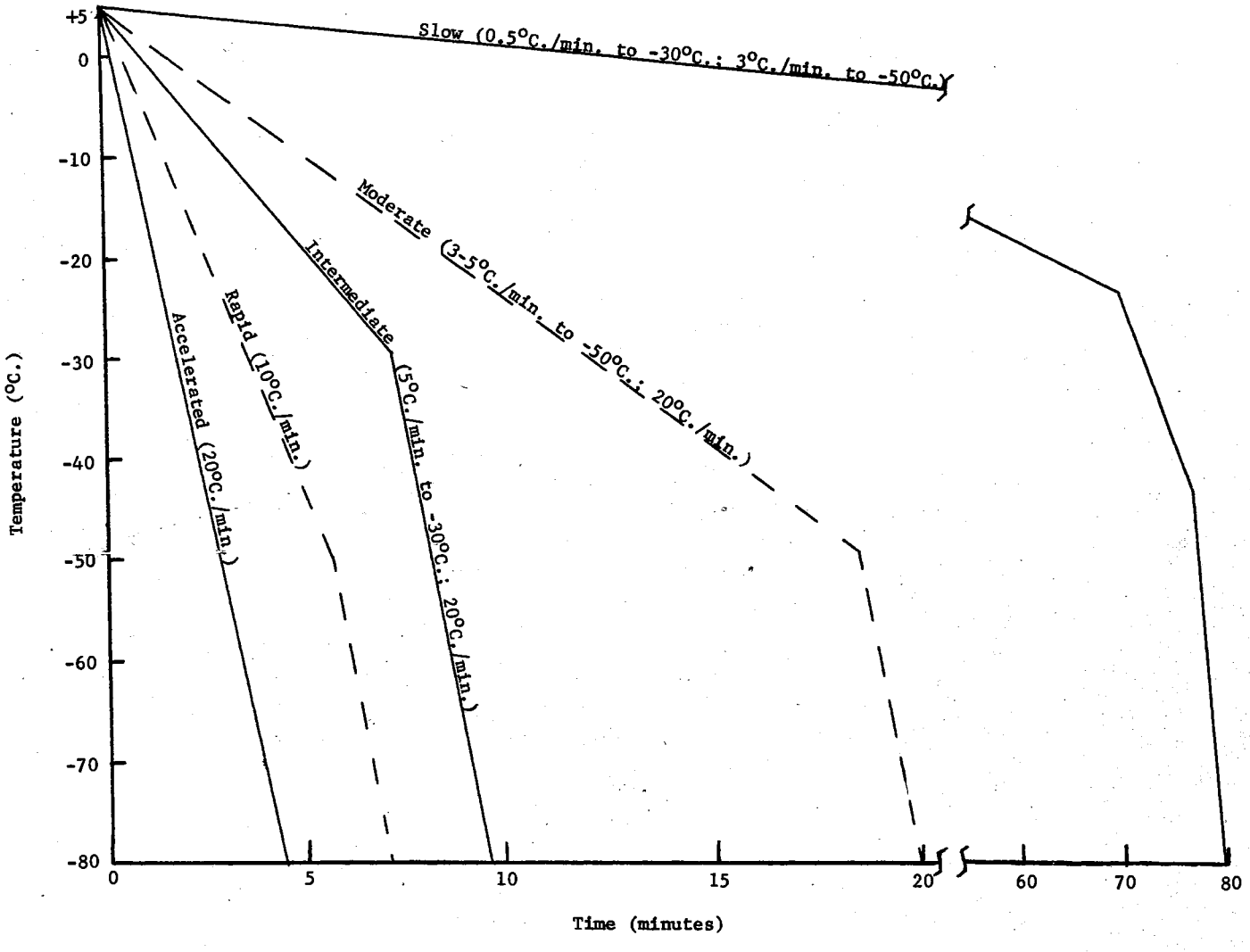

Figure 1. Freezing Bath Rate of Temperature Drop at 5 Rates of Freeze

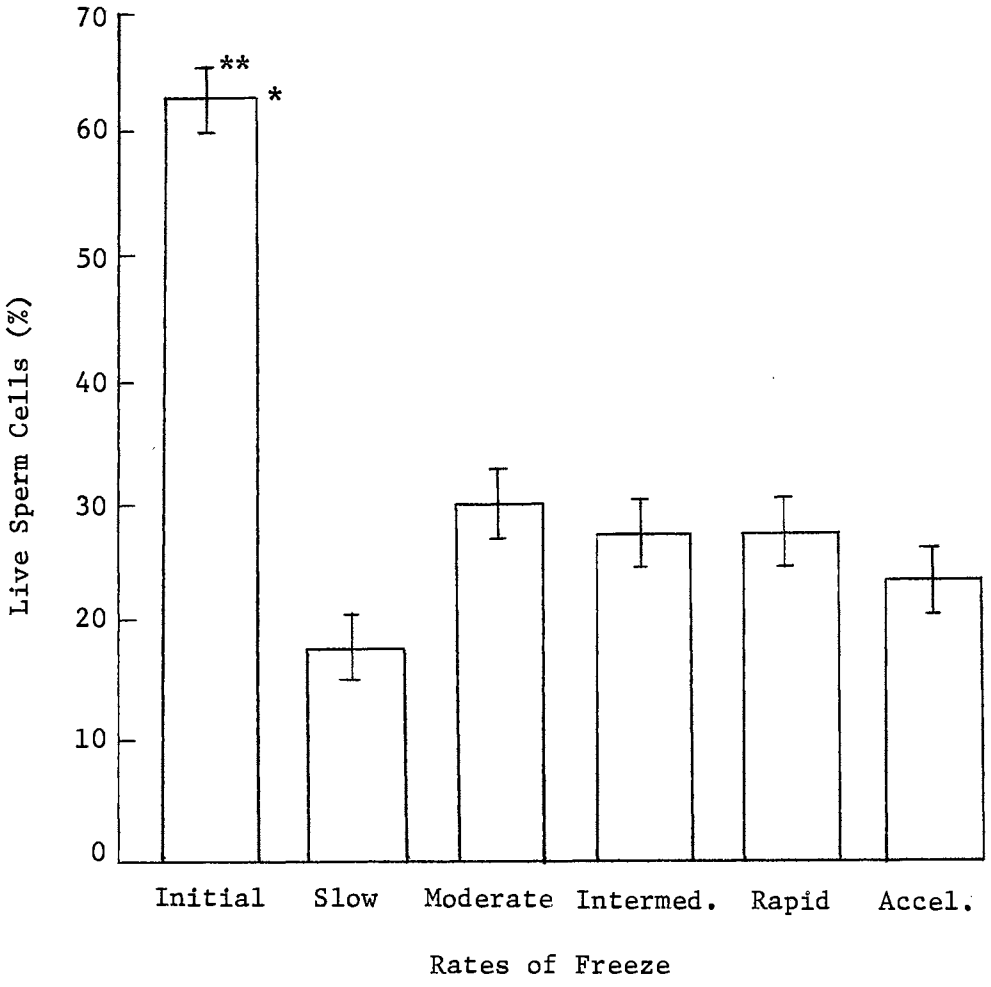

Figure 2. The Effect of Rate of Freeze on Percent Live Sperm Cells. (20 observations per mean).
*Means are in Appendix, Table III.
**Standard Error ± 2.8.

the percentage of live cells from the initial evaluations. The results of the analysis of variance of percent live evaluations in Table I show a highly significant difference ($P < .005$) among rates of freeze. The greatest sperm cell loss occurred in the slow rate of freeze (.5° C per minute) which resulted in 17.5% live post-freeze, significantly different ($P < .05$) from the initial evaluations and the average of all other rates of freeze. This indicated the slow rate is the most damaging rate of freeze. This is in agreement with VanDemark (1943) who compared .5° C per minute to 1° to 4° C per minute and found that the .5° C rate was inferior. The moderate rate (3° to 5° C per minute), shown in Figure 2, gave the highest percent live (29.7%) but was not significant from the other rates. There was a suggested trend in that as the rate of freeze increased from intermediate (5° C per minute to -30° C; 20° C per minute) to rapid (10° C per minute) to accelerated (20° C per minute), the percent live decreased respectively, 27.6%, 27.6%, 23.5%. In the moderate rate, the percent live cells after freeze was similar to those reported by Steinback and Foote (1964). These small differences could be of importance to A.I. organizations in increasing the utilization of superior sires.

The bull X date of collection interaction showed a highly significant effect ($P < .005$). This would suggest that the semen from different bulls responded differently on different dates. This indicates that each population of cells collected will react in a unique way that is dependent on the interplay of all the factors involved in production, storage and delivery of the cells. The bull X date of collection interaction indicates that bull and date have an effect on each other in their relation to rates of freeze.

TABLE I

ANALYSIS OF VARIANCE OF THE PERCENTAGE OF LIVE SPERM CELLS

Source of Variation	d.f.	Mean Squares	F	Level of Significance
Bulls	3	983.3	2.9	P < .1
Rates of Freeze	5	5154.7	60.3	P < .005
Dates Collected	4	951.6	2.7	P < .1
Bulls X Rates	15	85.5	1.4	
Bulls X Dates	12	336.8	5.6	P < .005
Rates X Dates	20	74.3	1.2	
Bulls X Rates X Dates	60	60.2		

The differences among bulls were not significant but did contribute to the variance ($P < .10$). Figure 3 represents the mean of five dates of collection for each of the four bulls and suggests that the higher the initial quality, the more successful the freezing operation will be regardless of rate.

The date of collection also contributed to the variation ($P < .10$) as is shown in Figure 4. There is the suggestion that on those days when semen quality is low, freezing operations are likely to be less successful than if quality was higher. This is in agreement with the observations in commercial freezing operations.

Percent Aged Acrosome Evaluation

Table II presents the analysis of variance for percent aged acrosomes using four bulls on five collection days with five different rates of freeze.

The interactions of bull X rate and bull X date are significant ($P < .005$). It is evident from the significant interactions in Table II that: (1) the semen from different bulls responded differently to various rates of freeze, (2) the semen from different bulls responded differently on different dates, and (3) different rates of freeze had different effects on different dates. These interactions reduce the emphasis that can be placed on the significance of the rates of freeze, bull and date effect. The interactions show the intricate relationship that rates of freeze, bulls, and dates of collection have on each other. This complexity of factors makes it imperative that further research be conducted to determine each factor's relative effect on the outcome of the freezing process.

TABLE II

ANALYSIS OF VARIANCE OF THE PERCENTAGE OF AGED ACROSOMES

Source of Variation	d.f.	Mean Squares	F	Level of Significance
Bulls	3	1013.9	4.6	P < .05
Rates of Freeze	5	1606.4	7.4	P < .005
Dates Collected	4	2407.1	10.9	P < .005
Bulls X Rates	15	216.2	2.6	P < .01
Bulls X Dates	12	221.2	2.6	P < .01
Rate X Dates	20	291.6	3.5	P < .005
Bulls X Rates X Dates	60	84.3		

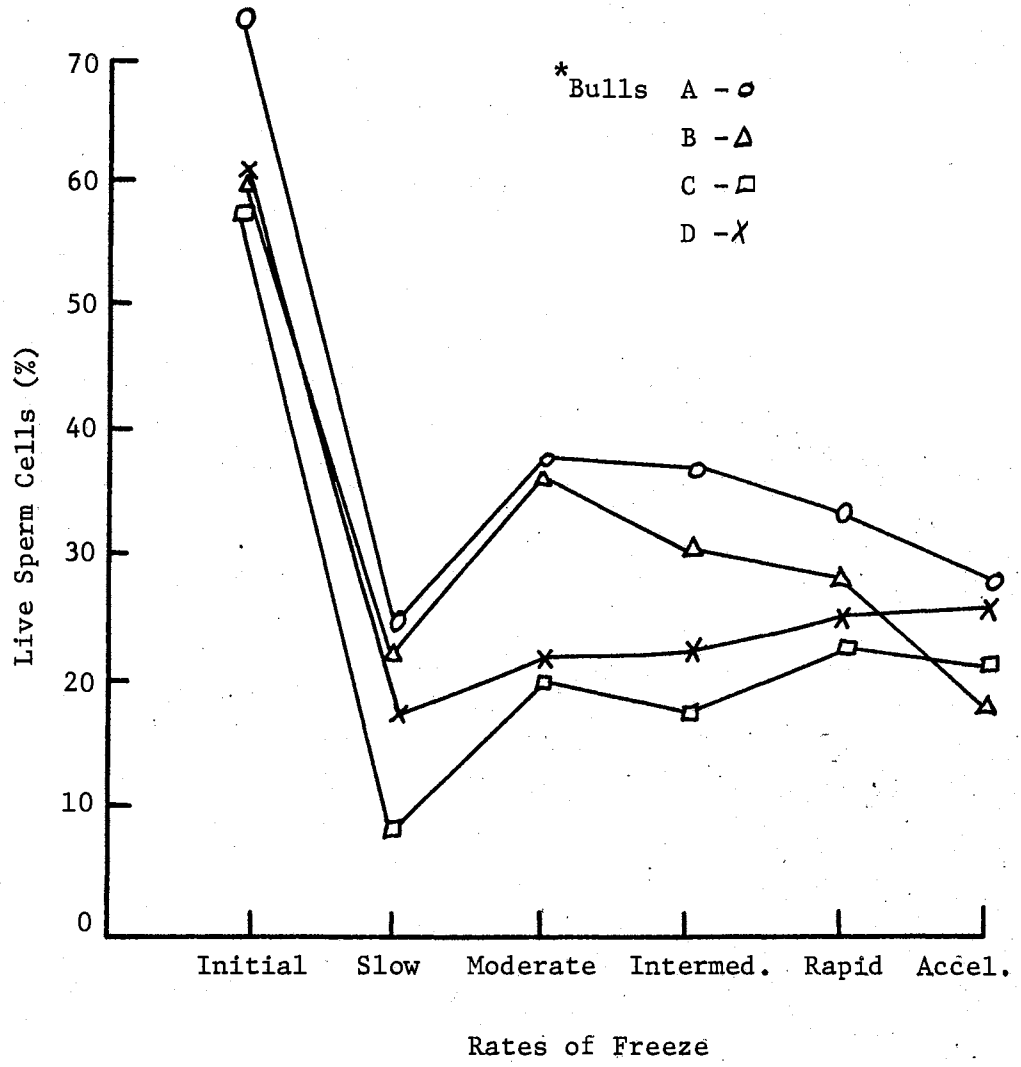

Figure 3. The Effect of Rate of Freeze, by Bulls, on Percent Live Sperm Cells. *Each plot is the average of 4 bulls (Appendix, Table IV).

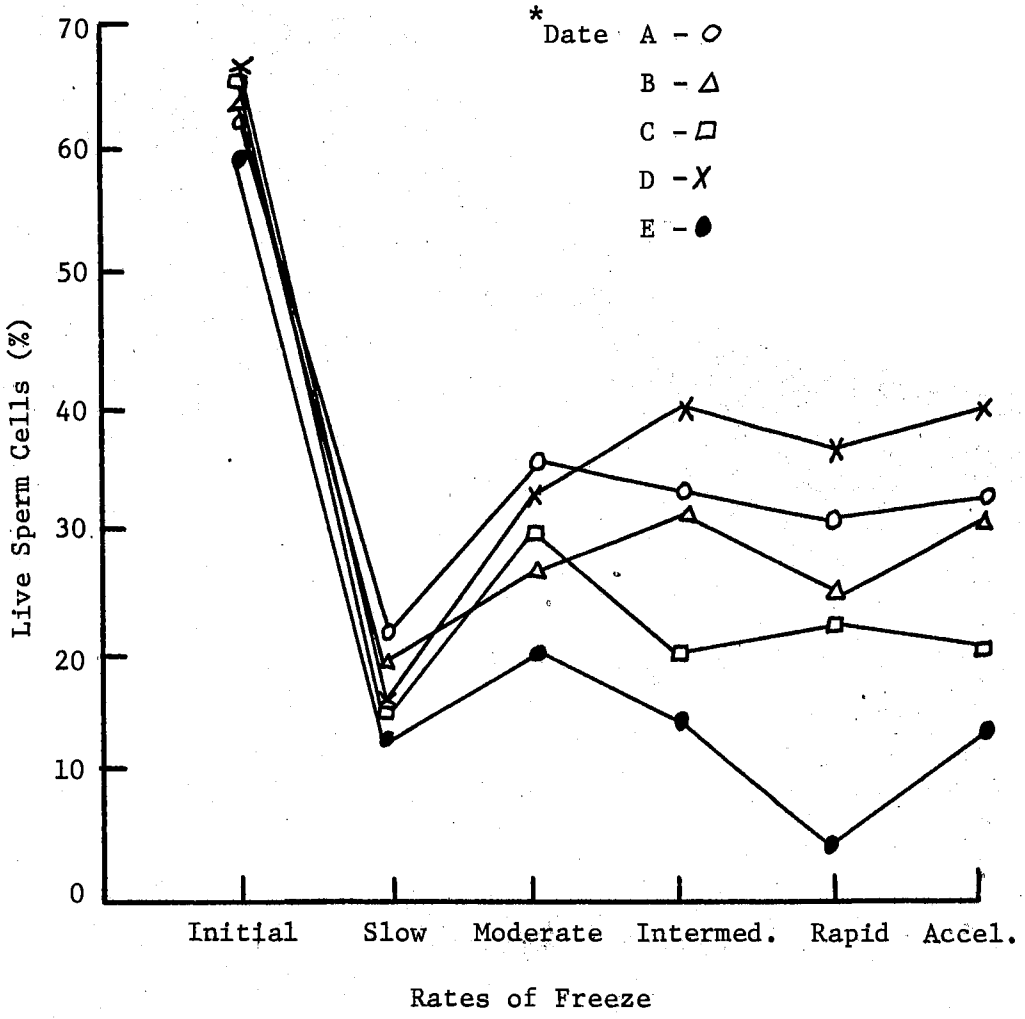

Figure 4. The Effect of Rate of Freeze, by Dates, on Percent Live Sperm Cells. *Each plot is the average of 4 bulls (Appendix, Table III).

The analysis indicated a highly significant effect ($P < .005$) of rate of freeze on the level of aged acrosomes. Figure 5 presents the difference in percent aged acrosomes for all rates of freeze. The initial percent aged acrosomes (21.2%) was significantly ($P < .05$) lower than all rates of freeze. The intermediate rate showed a higher percent aged acrosomes (45.3%) than the moderate, rapid, or accelerated rates. This was not the case in the percent live sperm cells evaluation. This indicates that freezing through the critical range at a moderate rate and then increasing the rate drastically, caused little change in the percent live characteristic. However, the acceleration after -30° C appears to increase the aging of the acrosome. The slow rate, though showing a high percent of aging with 45.1% aged acrosomes, was not significantly higher at the .05 level, than the rapid (39%), moderate (40.1%), and accelerated (41.6%) rates.

There is a significant difference ($P < .05$) among bulls indicating that the semen from some bulls responded differently to treatment. Figure 6 presents the means of the response of the ejaculates of the four bulls to treatment. Bull C was much more variable than were the other bulls.

The date of collection (Figure 7) showed a highly significant effect ($P < .005$), suggesting that the circumstances unique to the few days prior to and up to collection will cause different responses to treatment. A comparison of the magnitude of the mean square values for bulls and dates suggests that the state of the acrosome is highly sensitive to the conditions existing from one collection to the next. This is in good agreement with the findings of Awa (1970) that the acrosome is highly sensitive to changes in season, temperature or animal status.

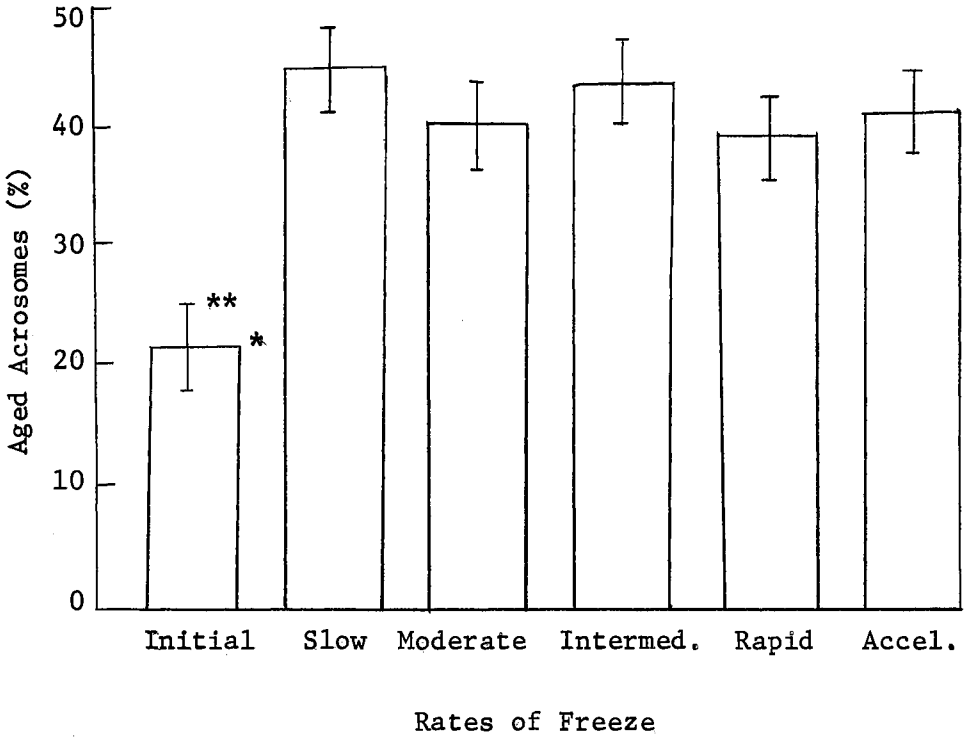

Figure 5. The Effect of Rate of Freeze on Percent Aged Acrosomes. (20 observations per mean).
*Means are in Appendix, Table VI.
**Standard Error ± 3.6.

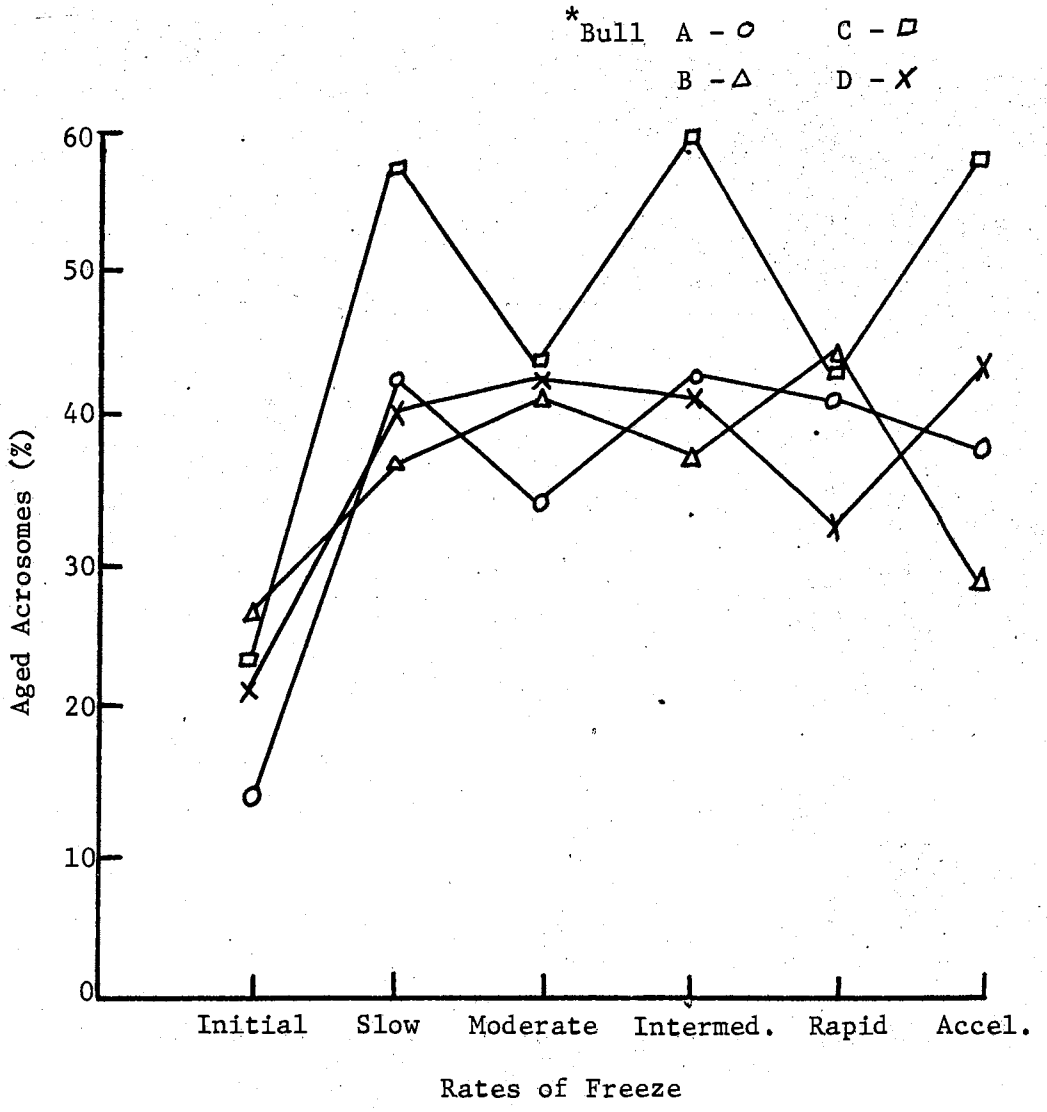

Figure 6. The Effect of Rate of Freeze, by Bulls, on Percent Aged Acrosomes. *Each plot is the average of 5 days of collection (Appendix, Table V).

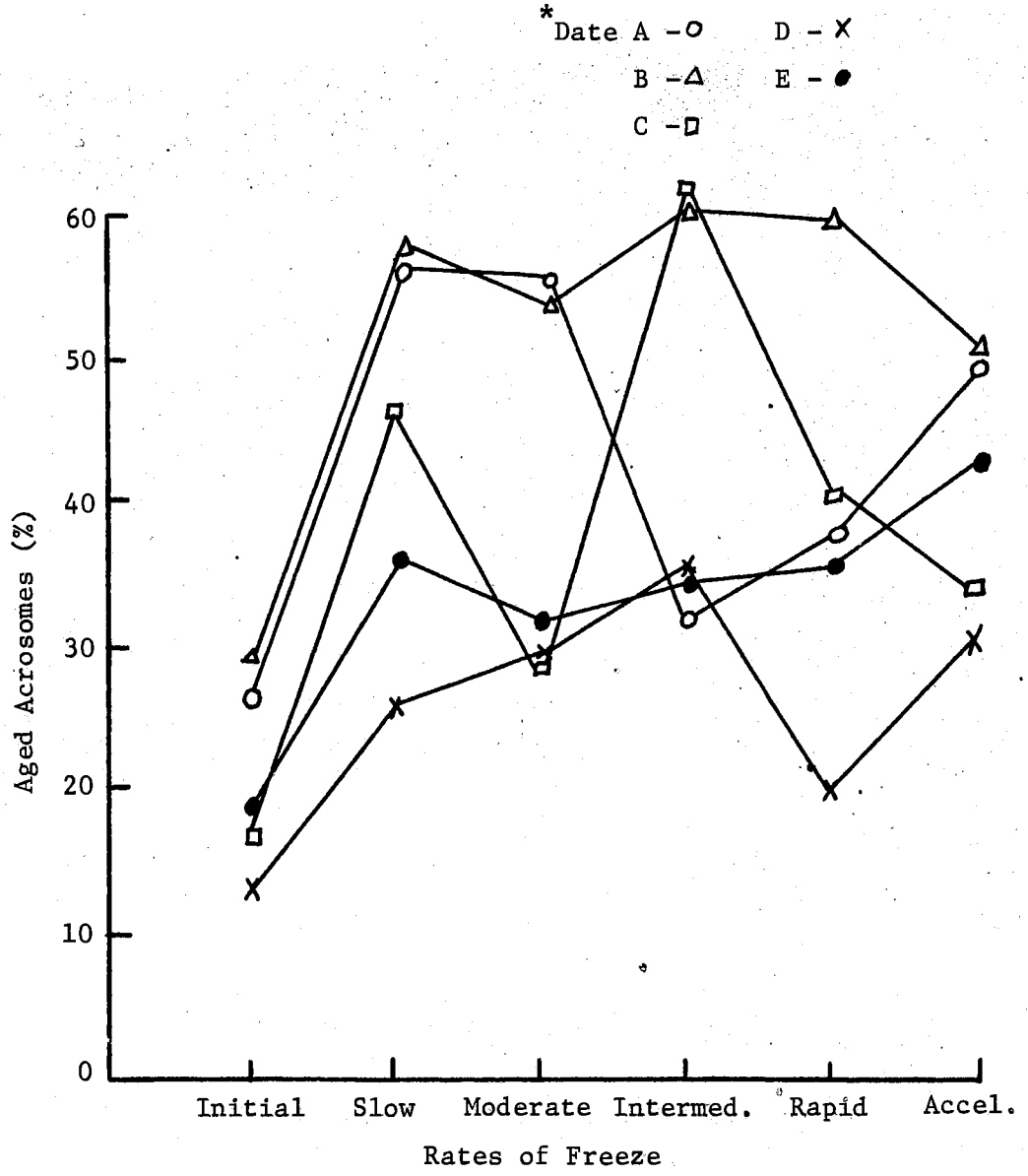

Figure 7. The Effect of Rate of Freeze, by Dates, on Percent Aged Acrosomes. *Each plot is the average of 4 bulls (Appendix, Table VI).

The two dates that had the highest initial percent aged acrosomes also gave the highest percent aged on the slow and moderate rates of freeze.

Although the data are limited, there is the suggestion that the lower the initial percentage of aged acrosomes, the more successful one will be with a moderate rate of freeze. Much more work needs to be done to define fully the relationship.

A comparison of Figures 6 and 7 with Figures 3 and 4, and the high degree of interactions in Table II, suggests that the acrosome is more sensitive to stress and changes than the percent live characteristic of an ejaculate. There appears to be more variation due to the date of collection in percent aged acrosome than percent live sperm cells.

The freezing and storage of semen is known to be a stress condition for the sperm cell. The percent live sperm cells and percent aged acrosomes were used in this experiment as evaluators of how well the sperm cells survived or measured the degree of damage from the stress imposed by the rates of freeze.

Even though there was considerable variation among bulls and dates, the study does indicate a highly significant effect ($P < .005$) of rate of freeze on both percent live and percent aged acrosomes.

This would suggest that the acrosome evaluation of frozen semen could be a useful measure of the success of the freezing procedure.

Graham, et al. (1971) working with boar semen suggested that the acrosome may be an important factor in successful freezing of semen. Pursel and Johnson (1971) found that the acrosome must be intact in order for boar spermatozoa to have fertilizing capacity after freezing. Acrosome evaluation may be a possible approach to use in further definition of freezing procedures and utilization patterns in the bull.

A more intensive study using more bulls collected on more days over a wider range of environmental differences is needed to best define the optimum rates of freeze. The relationship between acrosome integrity and fertility is not clear. A study to determine this relationship would aid in further development of the use of the acrosome as a method of sperm cell evaluation.

CHAPTER V

SUMMARY AND CONCLUSIONS

Four bulls were used in this study to determine the effect of rates of freeze on the state of the acrosome. Ejaculates were collected on five different days and a split-ejaculate study was conducted using five rates of freeze. The rates were slow (.5° C per minute), moderate (3° to 5° C per minute), intermediate (5° C per minute to -30° C; 20° C per minute), rapid (10° C per minute) and accelerated (20° C per minute).

The analysis of the data revealed a significant difference ($P < .005$) among rates of freeze for both the percent live and percent aged acrosome evaluations. There was also a significant difference ($P < .005$) of bull X date interaction in the percent live cells resulting from the rates of freeze. Significant differences ($P < .005$) in percent aged were found among bulls and among dates ($P < .005$). Bull X rate, bull X date ($P < .001$) and date X rate ($P < .005$) interactions also showed significant differences. It was evident from the significant bull interactions that the semen from different bulls respond differently to various rates of freeze. The expected stress of the rates of freeze was evident by the increase of percent aged and the decrease in percent live in all rates.

The initial ejaculate was significantly higher ($P < .05$) in percent live sperm cells and lower in percent aged acrosomes than the semen frozen at any of the rates. The slow rate of freeze suggested that

this was the most damaging rate to the sperm cells and state of the acrosome. The study also indicated the moderate rate was the most optimum rate for the preservation of sperm cell life and was acceptable in the maintenance of the condition of the acrosome.

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APPENDIXES

TABLE III

OVERALL AVERAGES OF PERCENT LIVE SPERM CELLS, BY DATE AND RATE OF FREEZE*

Rates of Freeze	Dates 1	2	3	4	5	Means
Initial Ejaculate	62.5**	63.8	63.8	65	60	63.0
Slow	20.8	19.3	16.5	17.8	13.0	17.5
Moderate	36.3	27.8	30.3	33.8	20.3	29.7
Intermediate	33.8	30.3	22.0	38.0	13.5	27.6
Rapid	32.5	30.8	27.8	31.3	15.8	27.6
Accelerate	30.5	25.0	22.8	34.8	4.3	23.5
Mean	36.0	32.8	30.5	36.8	21.1	

*Standard Error ± 3.4

**Means based on 4 observations per date

TABLE IV

OVERALL AVERAGES OF PERCENT LIVE SPERM CELLS, BY RATE OF FREEZE AND BULL*

Rates of Freeze	Bulls				Means
	A	B	C	D	
Initial Ejaculate	73.0**	60.0	58.0	61.0	63.0
Slow	23.8	21.2	9.0	15.8	17.5
Moderate	38.4	38.0	21.0	21.2	29.7
Intermediate	37.0	31.2	19.6	22.4	27.6
Rapid	32.4	29.8	22.6	25.6	27.6
Accelerated	26.6	19.2	21.8	26.2	23.5
Mean	38.5	33.2	25.3	28.7	

*Standard Error ± 3.6

**Means based on 5 observations per bull

TABLE V

OVERALL AVERAGES OF PERCENT AGED ACROSOME
BY RATE OF FREEZE AND BULL*

Rates of Freeze	Bulls A	B	C	D	Means
Initial Ejaculate	14.4**	26.6	23.4	20.4	21.2
Slow	43.2	37.8	58.6	40.8	45.1
Moderate	34.6	40.4	43.6	41.8	40.1
Intermediate	42.2	38.0	59.4	41.8	45.4
Rapid	40.2	44.0	40.8	31.0	39.0
Accelerate	34.8	28.6	58.4	44.4	41.6
Mean	34.9	35.9	47.4	36.7	

*Standard Error ± 3.6

**Means based on 5 observations per bull

TABLE VI

OVERALL AVERAGES OF PERCENT AGED ACROSOME BY DATE AND RATE OF FREEZE*

Rates of Freeze	Dates 1	2	3	4	5	Means
Initial Ejaculate	26.8**	29.5	17.8	13.0	19.0	21.2
Slow	57.3	57.5	47.3	26.3	37.3	45.1
Moderate	55.8	55.0	28.0	29.8	32.0	40.1
Intermediate	31.8	62.3	63.3	35.3	34.3	45.4
Rapid	37.3	60.0	41.8	20.0	36.0	39.0
Accelerated	50.0	50.3	34.0	30.3	43.3	41.6
Mean	43.1	52.4	38.7	25.8	33.6	

*Standard Error ± 2.8

**Means based on 4 observations per date

VITA

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